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# Declines in mitochondrial respiration during cardiac reperfusion: Age-dependent inactivation of α-ketoglutarate dehydrogenase

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**ABSTRACT** **We previously reported that cardiac reperfusion results in declines in mitochondrial NADH-linked respiration. The degree of inactivation increased with age and was paralleled by modification of protein by the lipid peroxidation product 4-hydroxy-2-nonenal. To gain insight into potential sites of oxidative damage, the present study was undertaken to identify specific mitochondrial protein(s) inactivated during ischemia and reperfusion and to determine which of these losses in activity are responsible for observed declines in mitochondrial respiration. Using a Langendorff rat heart perfusion protocol, we observed age-dependent inactivation of complex I during ischemia and complex IV and α-ketoglutarate dehydrogenase during reperfusion. Although losses in complex I and IV activities were found not to be of sufficient magnitude to cause declines in mitochondrial respiration, an age-related decrease in complex I activity during ischemia may predispose old animals to more severe oxidative damage during reperfusion. It was determined that inactivation of α-ketoglutarate dehydrogenase is responsible, in large part, for observed reperfusion-induced declines in NADH-linked respiration. α-Ketoglutarate dehydrogenase is highly susceptible to 4-hydroxy-2-nonenal inactivation *in vitro*. Thus, our results suggest a plausible mechanism for age-dependent, reperfusion-induced declines in mitochondrial function and identify α-ketoglutarate dehydrogenase as a likely site of free radical-mediated damage.**

Cardiac mitochondria exhibit declines in the rate of NADH-linked respiration and ATP synthesis as a result of myocardial ischemia and reperfusion (1–5). Loss in mitochondrial respiratory function because of reperfusion is more pronounced in senescent relative to adult heart (1). Because mitochondria are a site of free radical production and oxidative damage during reperfusion (6–9), it is likely that free radical events contribute to reperfusion-induced declines in mitochondrial function. The goals of this study were to (*i*) identify and distinguish between specific mitochondrial protein(s) inactivated as a result of ischemia and/or reperfusion; (*ii*) determine which of these losses in activity are responsible for observed declines in NADH-linked respiration; and (*iii*) evaluate the effects of age on these processes. Identification of enzymes inactivated during ischemia and reperfusion is necessary for rigorous assessment, at the molecular level, of events responsible for enzyme inactivation. Evaluation of which losses in activity are responsible for declines in the rate of overall mitochondrial respiration indicates the physiological consequences of enzyme inactivation. Distinguishing between ischemia- and reperfusion-induced declines in function will provide insight into factors contributing to free radical production and mitochondrial dysfunction. Thus, these studies are critical to elucidating mechanism(s) by which free radicals mediate declines in mitochondrial function during cardiac reperfusion.

We have previously demonstrated that reperfusion-induced declines in NADH-linked respiration are due to inhibition of electron transport and/or alterations in the supply of NADH (1). To distinguish between these possibilities and identify specific sites of mitochondrial dysfunction, hearts from 8- and 26-month-old rats were exposed to perfusion, ischemia, or ischemia/reperfusion by using a Langendorff perfusion protocol. After isolation of mitochondria, the rate of NADH-linked respiration and the activities of electron transport chain complexes I, III, and IV, glutamate dehydrogenase (GDH), and α-ketoglutarate dehydrogenase (KGDH) were determined. In addition, we performed *in vitro* studies with intact cardiac mitochondria by using specific inhibitors of enzymes critical to respiration to determine whether observed declines in certain activities were of sufficient magnitude to cause declines in NADH-linked respiration. The results of these experiments indicate that loss of mitochondrial respiratory activity during reperfusion is due, in large part, to molecular events that result in inactivation of KGDH. Identification of specific sites of ischemia- and reperfusion-induced loss in function suggests plausible mechanisms whereby free radicals contribute to age-dependent declines in mitochondrial respiration and provide direction for future studies designed to test these possibilities.

## MATERIALS AND METHODS

**Preparation and Perfusion of Isolated Rat Heart.** Hearts isolated from 8- and 26-month-old male Fisher-344 rats (National Institute of Aging colony) were perfused as described (1). Briefly, hearts were perfused in retrograde fashion according to Langendorff (10) with modified Krebs-Henseleit buffer (120 mM NaCl/4.8 mM KCl/2.0 mM $CaCl_2$/1.25 mM $MgCl_2$/1.25 mM $KH_2PO_4$/22 mM $NaHCO_3$/10 mM glucose) at 37°C, saturated with 95% $O_2$/5% $CO_2$. Experiments consisted of (*i*) a 90-min normoxic perfusion; (*ii*) a 25-min perfusion followed by a 25-min no-flow global ischemia; or (*iii*) a 25-min perfusion followed by a 25-min ischemia and then a 40-min reperfusion.

**Isolation of Mitochondria.** Subsarcolemmal mitochondria were isolated from ventricles as reported (1). Ventricles were homogenized in 180 mM KCl/5.0 mM Mops/2.0 mM EGTA, pH 7.25 (20 ml/g tissue) with a Polytron homogenizer (low setting, 3 s). The homogenate was then centrifuged at 500 × *g* for 7.5 min at 4°C. The supernatant was filtered through cheese cloth and centrifuged at 5,000 × *g* for 10 min at 4°C. The resulting mitochondrial pellet was washed twice and resuspended into 150 μl of homogenization buffer to a final protein concentration of ≈25 mg/ml. Protein determinations were made by using the BCA method (Pierce), with BSA as a standard. Mitochondria were kept at 4°C before various anal-

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Abbreviations: HNE, 4-hydroxy-2-nonenal; KGDH, α-ketoglutarate dehydrogenase; GDH, glutamate dehydrogenase.
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yses and exhibited no change in state 3 or state 4 respiratory rates for up to 3.0 h.

**Evaluation of Mitochondrial $O_2$ Consumption.** ADP-independent (state 4) and -dependent (state 3) respiration were measured by using a Clark-type oxygen electrode (Instech, Plymouth Meeting, PA) (1). Mitochondria were diluted to a protein concentration of 0.5 mg/ml in respiration buffer (120 mM KCl/5.0 mM $KH_2PO_4$/5.0 mM Mops/1.0 mM EGTA, pH 7.25). State 2 respiration was initiated by the addition of glutamate (15 mM). After 2.0 min, state 3 respiration was initiated by addition of ADP (0.5 mM). On depletion of ADP, state 4 respiration was monitored.

**Electron Transport Chain Assays.** Electron transport chain assays were performed as in refs. 11 and 12. For analysis of complex I activity, mitochondria were diluted into a buffer containing 35 mM $KH_2PO_4$, 5.0 mM $MgCl_2$, and 2.0 mM NaCN at pH 7.25 and sonicated for 30 s at setting 3 (Branson Sonifier 450). Complex I was then assayed by monitoring the consumption of NADH (Hewlett-Packard model 8453 diode array spectrophotometer) at 340 nm ($\varepsilon$ = 6,200 $M^{-1}\cdot cm^{-1}$) on addition of 5.0 $\mu$M of antimycin A, 60 $\mu$M ubiquinone-1 (donated by Eisai, Tokyo), and 75 $\mu$M NADH to 25 $\mu$g/ml mitochondrial protein. For analysis of complex III activity, mitochondria were diluted into a buffer containing 35 mM $KH_2PO_4$, 5.0 mM $MgCl_2$, 2.0 mM NaCN, and 0.5 mM EDTA at pH 7.25 and sonicated for 30 s at setting 3 (Branson Sonifier 450). Complex III activity was then measured as the initial rate of the reduction of cytochrome *c* at 550 nm ($\varepsilon$ = 18,500 $M^{-1}\cdot cm^{-1}$) on addition of 40 $\mu$M reduced decylubiquinone and 50 $\mu$M cytochrome *c* to 2.5 $\mu$g/ml mitochondrial protein. Addition of 2 $\mu$g of antimycin A completely inhibited reduction of cytochrome *c*. For analysis of complex IV, mitochondria were diluted into a hypotonic buffer containing 20 mM $KH_2PO_4$ (pH 7.25) followed by sonication for 10 s in a sonicator bath (Fisher). Complex IV was assayed polarographically by the rate of oxygen consumption on addition of 5.0 mM ascorbate, 250 $\mu$M *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenyldiamine (TMPD), and 10 $\mu$M cytochrome *c* to 100 $\mu$g/ml mitochondrial protein. All assays were performed at room temperature.

**Dehydrogenase Assays.** Dehydrogenase assays (11, 13) were performed at room temperature with sonicated mitochondria (30 s, setting 3, Branson Sonifier 450) in a buffer containing 35 mM $KH_2PO_4$, 5.0 mM $MgCl_2$, 2.0 mM NaCN, and 0.5 mM EDTA at pH 7.25. Glutamate dehydrogenase was assayed by measuring the oxidation of NADH at 340 nm on the addition of 50 $\mu$M NADH, 50 mM $NH_4Cl$, and 15 mM $\alpha$-ketoglutarate to 50 $\mu$g/ml mitochondrial protein. $\alpha$-Ketoglutarate dehydrogenase was assayed by measuring the reduction of $NAD^+$ at 340 nm on the addition of 0.5 mM $NAD^+$, 200 $\mu$M thiamine pyrophosphate chloride (TPP), 40 $\mu$M CoASH, and 2.0 mM $\alpha$-ketoglutarate to 25 $\mu$g/ml mitochondrial protein. All $NAD^+$/NADH-linked assays were done in the presence of 2.5 $\mu$M rotenone to prevent NADH consumption by complex I.

***In Vitro* Inactivation of Complex I, Complex IV, and State 3 Respiration.** To determine the degree of inactivation of complex I and IV required to cause declines in the rate of state 3 respiration, various concentrations of electron transport chain inhibitors were administered, and state 3 respiration and complex activities were measured as described (14). Mitochondria (0.5 mg/ml) were incubated for 5.0 min in respiration buffer containing 15 mM glutamate and rotenone (0–240 nM) or NaCN (0–256 $\mu$M) to inhibit complexes I and IV, respectively. For experiments addressing inhibition of complex I, after incubation with rotenone, 25 mM ADP was added, and the rate of state 3 respiration was determined as described above. Mitochondria were then diluted to 50 $\mu$g/ml, and complex I activity was measured. Dilution did not affect the degree of rotenone inactivation of complex I. In experiments involving treatment with $CN^-$, mitochondria were diluted after 5.0 min to 0.25 mg/ml in either respiration buffer for state 3 measurements or 20 mM $K_2HPO_4$, pH 7.25 to determine complex IV activity. Incubation of mitochondria with rotenone or $CN^-$ for times greater than 5.0 min did not result in further inactivation under the conditions of these experiments.

## RESULTS

To accurately assess the relationship between the degree of inactivation of specific enzymes and the fractional loss in the rate of mitochondrial respiration, it was important to begin this study by establishing relative declines in NADH-linked respiratory rates caused by ischemia and reperfusion as a function of age. Hearts excised from adult (8-month) and senescent (26-month) rats were exposed to perfusion (90 min), ischemia (25 min), or ischemia/reperfusion (25 min/40 min) as described in *Materials and Methods*. Mitochondria were then isolated, and the rate of NADH-linked ADP-dependent (state 3) respiration was assessed. In general agreement with our previous study (1), mitochondria from perfused hearts of both age groups exhibited nearly identical respiratory rates (Table 1). Ischemia (25 min) resulted in an ≈18% loss in the rate of state 3 respiration independent of age. In contrast to ischemia, decreases in mitochondrial respiratory rates as a result of reperfusion were dependent on age. Reperfusion led to further 15.0% and 28.5% declines in the rate of state 3 respiration for hearts from 8- and 26-month-old rats, respectively (Table 1). Fractional losses in respiratory activity because of ischemia (perfusion minus ischemic values) and reperfusion (ischemic minus reperfusion values) are expressed relative to age-matched perfusion values. In addition, the duration of perfusion (0–90 min) had no effect on the rate of NADH-linked mitochondrial respiration or enzyme activities evaluated.

In an attempt to determine whether inactivation of electron transport leads to observed declines in NADH-linked respiration, complexes I, III, and IV activities were measured in mitochondria isolated from hearts exposed to perfusion, ischemia, or ischemia/reperfusion. No age-related differences in complex I, III, and IV

Table 1. Effects of ischemia and reperfusion on state 3 respiration and complex activities

| Protocol | *n* | State 3, nmol of O·min$^{-1}$·mg$^{-1}$ | Complex I, nmol of NADH·min$^{-1}$·mg$^{-1}$ | Complex III, nmol of cytochrome *c*·min$^{-1}$·mg$^{-1}$ | Complex IV, nmol of O·min$^{-1}$·mg$^{-1}$ |
|---|---|---|---|---|---|
| 8 month old | | | | | |
| Perfused | 7 | 203.5 ± 26.2* | 136.0 ± 24.5** | 1,921.5 ± 321.3 | 1,993.0 ± 246.7 |
| Ischemic | 6 | 167.0 ± 20.3*‡ | 117.8 ± 6.3**‡‡ | 2,205.4 ± 376.9 | 1,915.5 ± 169.7 |
| Reperfused | 6 | 136.5 ± 22.6‡¶ | 119.7 ± 8.0 | 1,921.5 ± 322.4 | 2,076.3 ± 412.4¶¶ |
| 26 month old | | | | | |
| Perfused | 6 | 204.2 ± 26.2† | 131.5 ± 17.2†† | 1,969.3 ± 248.2 | 2,019.4 ± 327.9 |
| Ischemic | 6 | 168.3 ± 26.6†§ | 99.5 ± 10.2††‡‡ | 2,195.0 ± 430.0 | 2,135.0 ± 309.5§§ |
| Reperfused | 6 | 110.1 ± 11.7¶§ | 97.3 ± 10.7 | 2,063.6 ± 298.4 | 1,709.4 ± 52.4§§¶¶ |

Perfusion, ischemia, and reperfusion protocols were performed as described in *Materials and Methods*. Values are expressed as the mean ± SD. *P* values (determined from paired *t* test) where like symbols indicate values compared. State 3: *, ≤0.009; †, ≤0.02; ‡, ≤0.02; §, ≤0.0003; ¶, ≤0.02. Complex I: **, ≤0.05; ††, ≤0.001; ‡‡, ≤0.002. Complex IV: §§, ≤0.004; ¶¶, ≤0.03.

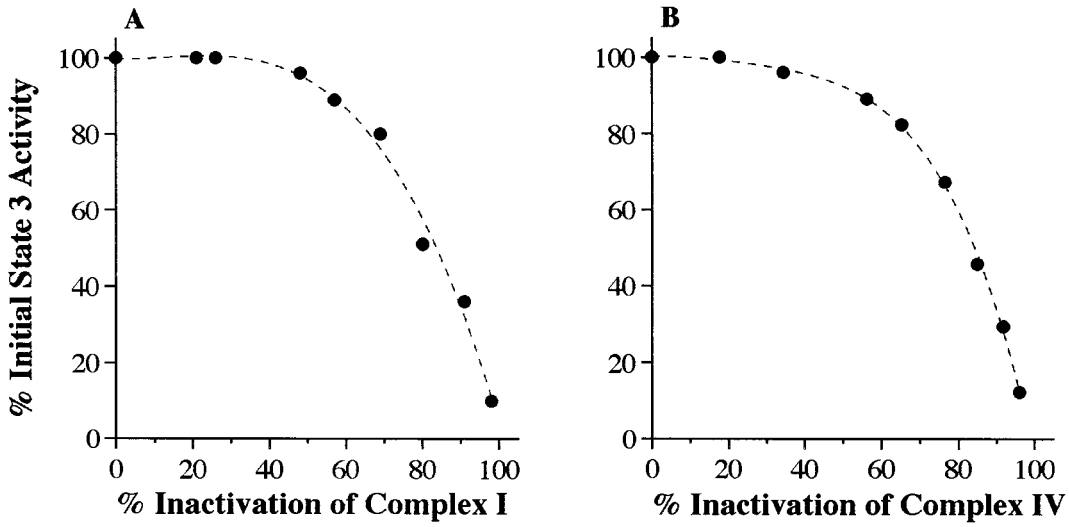


FIG. 1. Relationship between degree of inactivation of complexes I and IV and loss of state 3 respiration. Mitochondria (0.5 mg/ml) were incubated in the presence of 15 mM glutamate with (*A*) rotenone (0–240 nM) or (*B*) NaCN (0–256 $\mu$M). After 5.0 min, the rate of state 3 respiration and complex I or complex IV activity were measured as described in *Materials and Methods*.

activities were observed in mitochondria isolated from perfused hearts (Table 1). Ischemia resulted in a significant drop in complex I activity, but no change in the activity of either complex III or IV. The degree of ischemia-induced complex I inactivation depended on age, with a 13.4% and 24.3% decline in activity in mitochondria isolated from adult and senescent hearts, respectively. No further loss in complex I activity was observed during reperfusion. For mitochondria from hearts of either age group, the activity of complex III was not significantly altered by reperfusion. In contrast, reperfusion resulted in an age-dependent decline in complex IV activity. No loss was observed in hearts from 8-month-old rats, whereas a 19.9% loss in activity was seen in hearts from 26-month-old rats relative to ischemic values (Table 1). The age-dependent inactivation of complex I during ischemia and complex IV during reperfusion did not reflect the respective losses in state 3 respiration (Table 1). These observations call into question the contributions of complex I and IV inactivation to observed declines in NADH-linked state 3 respiration.

To determine whether observed declines in complexes I and IV activities during ischemia and reperfusion (Table 1) could, at least in part, account for loss in NADH-linked state 3 respiration, mitochondria were isolated from nonperfused rat heart and treated with various concentrations of specific inhibitors of complex I (rotenone) or complex IV ($CN^-$). The degree of complex inactivation required to cause declines in the rate of state 3 respiration was then determined. As shown in Fig. 1, complex I or IV activities could be inhibited up to 50% with minimal effects on the rate of mitochondrial respiration. Further inactivation of complex I or IV resulted in significant declines in state 3 respiration (Fig. 1). It is therefore apparent that complexes I and IV are not rate-limiting, and that observed declines in the maximum rates of these enzymes because of ischemia and reperfusion are not of sufficient magnitude (Table 1) to elicit declines in NADH-linked respiration.

Based on results presented in Fig. 1, we conclude that supply of NADH to the electron transport chain controls the rate of NADH-linked mitochondrial respiration. Alterations in the rate of NADH synthesis would therefore have profound effects on respiratory activity. For these reasons, the activities of GDH and KGDH were measured in mitochondria isolated from hearts exposed to perfusion, ischemia, or ischemia/reperfusion. These dehydrogenases were chosen because the activities of both enzymes are required for the synthesis of NADH from glutamate (13), the respiratory substrate used in this study, and because KGDH has been shown to be highly susceptible to free radical-mediated inactivation (11, 13, 15). As shown in Table 2, no significant age-related difference in either enzyme activity was apparent in mitochondria isolated from perfused hearts. In addition, GDH exhibited no appreciable alterations in activity during ischemia or reperfusion for either age group. Ischemia also was found to have no effect on KGDH activity. Reperfusion, however, led to a significant decline in KGDH activity in hearts isolated from both adult and senescent animals. The degree of inactivation depended on age. KGDH exhibited a 13.0% and 25.2% loss in activity in hearts from 8- and 26-month-old rats, respectively (Table 2), reflecting the fractional loss in NADH-linked respiration observed during reperfusion (Table 1). We have recently shown that selective inactivation of KGDH in isolated cardiac mitochondria results in a loss in NADH-linked respiration (13). Unlike complexes I and IV, the degree of KGDH inactivation was found to be directly proportional to the degree of respiratory inhibition (13). Therefore, age-dependent inactivation of KGDH during reperfusion (Table 2) is, in large part, responsible for observed declines in mitochondrial respiration (Table 1).

## DISCUSSION

Cardiac ischemia and reperfusion result in declines in the maximum rate of NADH-linked mitochondrial respiration (1–5). The degree of reperfusion-induced inhibition increases

Table 2. Effects of ischemia and reperfusion on GDH and KGDM activities

| Protocol | *n* | GDH | KGDH |
|---|---|---|---|
| 8-month old | | | |
| Perfused | 7 | 38.4 ± 4.9 | 98.0 ± 16.9 |
| Ischemic | 6 | 36.5 ± 4.0 | 99.6 + 3.5* |
| Reperfused | 6 | 35.4 ± 2.7 | 86.7 + 8.9*‡ |
| 26-month old | | | |
| Perfused | 6 | 36.0 ± 6.8 | 95.9 ± 2.7 |
| Ischemic | 6 | 35.9 ± 3.6 | 94.7 ± 11.0† |
| Reperfused | 6 | 34.8 ± 5.3 | 70.8 ± 8.4†‡ |

Perfusion, ischemia, and reperfusion protocols were performed as described in *Materials and Methods*. Enzyme activities are expressed as nmol of $NADH \cdot min^{-1} \cdot mg^{-1}$ and values represent the mean ± SD. *P* values (determined from paired *t* test) where like symbols indicate values compared. KGDH: *, ≤0.005; †, ≤0.001; ‡, ≤0.007.

in an age-dependent fashion (Table 1) (1). The present study was undertaken to identify specific mitochondrial protein(s) inactivated during ischemia and reperfusion, and to determine which of these losses in activity are responsible for declines in mitochondrial respiration. We observed selective inactivation of complex I during ischemia and complex IV and KGDH during reperfusion. Inactivation of each enzyme was more pronounced in hearts from senescent (26-month-old) relative to adult (8-month-old) rats (Tables 1 and 2). Ischemia- or reperfusion-induced loss of mitochondrial respiration could not be attributed to decreases in the activity of complex I or IV, because these enzymes must be inactivated to magnitudes greater than those observed in this study to cause declines in mitochondrial respiratory rates (Fig. 1). In contrast, *in vitro* experiments revealed that selective inhibition of KGDH leads to a loss in NADH-linked respiration, the magnitude of which is directly proportional to the degree of KGDH inactivation (13). In the current study, loss in KGDH activity because of reperfusion reflected age-dependent declines in mitochondrial respiration (Table 1 and 2). Thus, inactivation of KGDH is responsible, in large part, for reperfusion-induced declines in NADH-linked respiration. It has been proposed that free radical events play a critical role in myocardial reperfusion injury (1, 6–9, 16–21). Identification of specific mitochondrial enzymes inactivated during ischemia and reperfusion provides insight into factors that may promote free radical generation and potential mechanisms by which free radicals mediate age-dependent, reperfusion-induced loss of mitochondrial respiratory function.

Mitochondria isolated from hearts exposed to ischemia exhibit elevated rates of free radical generation when allowed to respire (22, 23). Ischemia-induced alterations in certain mitochondrial functions therefore predispose these organelles to free radical production during reoxygenation. Of the respiratory complexes and dehydrogenases evaluated, only complex I lost activity as a result of ischemia (Table 1). Furthermore, the degree of inactivation increased with age. Although observed declines in complex I activity are not directly responsible for loss in mitochondrial respiration during ischemia (Fig. 1), this process may play a role in subsequent reperfusion damage. Inhibition of complex I in isolated mitochondria has been shown to promote free radical production (24, 25). Loss of complex I activity during ischemia would therefore be expected to result in increased free radical generation during reperfusion. The greater degree of complex I inactivation in hearts from senescent relative to adult rats (Table 1) may be responsible for age-dependent increases in free radical generation and oxidative damage because of reperfusion (1). It should be noted that, whereas the ratio of $NADH/NAD^+$ increases with the duration of ischemia, the total pool of nicotinamide-adenine dinucleotides has been reported to decline (26, 27). Supply of NADH to the electron transport chain appears to be the rate-determining step in mitochondrial respiration (Fig. 1; refs. 11, 13, and 28–32). Therefore, loss of nicotinamide-adenine dinucleotides may be responsible for observed declines in NADH-dependent respiration during ischemia (Table 1).

Results of *in vivo* (1, 16, 19) and *in vitro* (11, 13) studies suggest that peroxidation of membrane lipids is involved in reperfusion-induced inactivation of KGDH and NADH-linked mitochondrial respiration. Cardiac reperfusion is associated with increases in lipid peroxidation (19), production of 4-hydroxy-2-nonenal (HNE) (16), and modification to mitochondrial protein by HNE (1). HNE is a major product of lipid peroxidation that readily reacts with and inactivates protein (33–37). As demonstrated in *in vitro* studies, treatment of intact cardiac mitochondria with micromolar concentrations of HNE causes rapid declines in the rate of NADH-linked respiration (13). This was because of selective inactivation of KGDH. HNE had no effect on electron transport complexes I-IV or GDH (11, 13). Thus, the *in vitro* effects of HNE on KGDH and NADH-linked respiration mirror the effects of reperfusion on cardiac mitochondria. HNE may therefore be an important mediator of free radical damage during cardiac reperfusion, and KGDH is a likely site of HNE-induced inactivation.

*In vitro* inactivation of KGDH by HNE involves modification of essential lipoic acid residues covalently linked to E2 subunits of the enzyme (11, 13). Lipoic acid is a strong hydrophobic nucleophile, making KGDH a highly susceptible target for interaction with HNE, a hydrophobic electrophile. In addition, the two sulfur atoms on lipoic acid cycle between reduced and oxidized states during enzyme catalysis (38). The reduced state is required for reaction with HNE, a condition satisfied when the reduction potential ($NADH/NAD^+$) is high (11). During ischemia, the ratio of $NADH/NAD^+$ increases because of the absence of $O_2$ as a terminal electron acceptor (39, 40). KGDH is therefore a prime candidate for inactivation on reoxygenation and subsequent lipid peroxidation. Direct evidence for this mechanism requires detection of HNE-modified lipoic acid residues on KGDH. Antibodies to cysteine–, histidine–, and lysine–HNE adducts were previously used to demonstrate the occurrence of HNE-modified protein during reperfusion (1). However, *in vitro* studies indicate that these antibodies do not cross-react with lipoic acid–HNE adducts known to be present in HNE-treated mitochondria (11). Clearly, identification of KGDH as an enzyme that both loses activity during reperfusion and is highly susceptible to free radical-induced inactivation provides direction for the design of experimental strategies for testing the involvement of free radicals in reperfusion injury. In addition, pyruvate dehydrogenase, an enzyme that shares structural and functional similarities with KGDH and contains covalently bound lipoic acid residues (38), may exhibit a similar response to reperfusion and therefore deserves attention (11).

In this study, reperfusion-induced losses in mitochondrial respiration were greater than previously observed (15.0% vs. 0% for adult heart and 28.5% vs. 22.7% for senescent heart), particularly for hearts from adult (8-month-old) rats (1). Age-dependent differences in the degree of dysfunction were therefore smaller than reported earlier (1) but highly significant (Table 1). Under basal conditions, the functional parameters tested did not vary with age, indicating that subtle alterations are likely to affect the response to stress and may account for variability between studies (Table 1; ref. 1). To define factors that contribute to increases in the susceptibility to reperfusion damage, it is important to conduct future investigations by using hearts from animals of a broad range of age groups exposed to a variety of conditions of ischemia and reperfusion. The significance of this study is that specific sites of enzymatic dysfunction that play a role in loss in mitochondrial respiration during reperfusion have been identified. This information allows for rigorous evaluation of free radical mechanisms of damage, when in the sequence of ischemia and reperfusion certain critical alterations take place, and how these parameters vary with age.

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1. Lucas, D. T. & Szweda, L. I. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 510–514.
2. van Jaarsveld, H., Kuyl, J. M., Alberts, D. W. & Wiid, M. N. (1994) *Res. Commun. Mol. Pathol. Pharmacol.* **85,** 33–44.
3. Hardy, L., Clark, J. B., Darley-Usmar, V. M., Smith, D. R. & Stone, D. (1991) *Biochem. J.* **274,** 133–137.
4. Lesnefsky, E. J., Tandler, B., Ye, J., Slabe, T. J., Turkaly, J. & Hoppel, C. L. (1997) *Am. J. Physiol.* **273,** H1544–H1554.
5. Duan, J. & Karmazyn, M. (1989) *Can. J. Physiol. Pharmacol.* **67,** 704–709.
6. Ambrosio, G., Zweier, J. L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritto, I., Cirillo, P., Condorelli, M., Chiariello, M., *et al.* (1993) *J. Biol. Chem.* **268,** 18532–18541.

7. Das, D. K., George, A., Liu, X. K. & Rao, P. S. (1989) *Biochem. Biophys. Res. Commun.* **165,** 1004–1009.
8. Ferrari, R. (1996) *J. Cardiovasc. Pharmacol.* **28,** S1–10.
9. Ambrosio, G., Flaherty, J. T., Duilio, C., Tritto, I., Santoro, G., Elia, P. P., Condorelli, M. & Chiariello, M. (1991) *J. Clin. Invest.* **87,** 2056–2066.
10. Langendorff, O. (1895) *Arch. Gesamte Physiol. Mens. Tiere* **61,** 291–332.
11. Humphries, K. M. & Szweda, L. I. (1998) *Biochemistry* **37,** 15835–15841.
12. Veitch, K., Hombroeckx, A., Caucheteux, D., Pouleur, H. & Hue, L. (1992) *Biochem. J.* **281,** 709–715.
13. Humphries, K. M., Yoo, Y. & Szweda, L. I. (1998) *Biochemistry* **37,** 552–557.
14. Davey, G. P. & Clark, J. B. (1996) *J. Neurochem.* **66,** 1617–1624.
15. Rokutan, K., Kawai, K. & Asada, K. (1987) *J. Biochem. (Tokyo)* **101,** 415–422.
16. Blasig, I. E., Grune, T., Schonheit, K., Rohde, E., Jakstadt, M., Haseloff, R. F. & Siems, W. G. (1995) *Am. J. Physiol.* **268,** H14–H22.
17. Bolli, R., Patel, B. S., Jeroudi, M. O., Lai, E. K. & McCay, P. B. (1988) *J. Clin. Invest.* **82,** 476–485.
18. Garlick, P. B., Davies, M. J., Hearse, D. J. & Slater, T. F. (1987) *Circ. Res.* **61,** 757–760.
19. Kramer, J. H., Misik, V. & Weglicki, W. B. (1994) *Ann. N.Y. Acad. Sci.* **723,** 180–196.
20. McCord, J. M. (1988) *Free Radical Biol. Med.* **4,** 9–14.
21. Zweier, J. L. (1988) *J. Biol. Chem.* **263,** 1353–1357.
22. Otani, H., Tanaka, H., Inoue, T., Umemoto, M., Omoto, K., Tanaka, K., Sato, T., Osako, T., Masuda, A., Nonoyama, A., *et al.* (1984) *Circ. Res.* **55,** 168–175.
23. Ueta, H., Ogura, R., Sugiyama, M., Kagiyama, A. & Shin, G. (1990) *J. Mol. Cell. Cardiol.* **22,** 893–899.
24. Kwong, L. K. & Sohal, R. S. (1998) *Arch. Biochem. Biophys.* **350,** 118–126.
25. Boveris, A. (1984) *Methods Enzymol.* **105,** 429–435.
26. Jennings, R. B., Reimer, K. A., Hill, M. L. & Mayer, S. E. (1981) *Circ. Res.* **49,** 892–900.
27. Klein, H. H., Schaper, J., Puschmann, S., Nienaber, C., Kreuzler, H. & Schaper, W. (1981) *Basic Res. Cardiol.* **76,** 612–621.
28. Cooney, G. J., Taegtmeyer, H. & Newsholme, E. A. (1981) *Biochem. J.* **200,** 701–703.
29. Moreno-Sanchez, R., Hogue, B. A. & Hansford, R. G. (1990) *Biochem. J.* **268,** 421–428.
30. Balaban, R. S. (1990) *Am. J. Physiol.* **258,** C377–389.
31. Brown, G. C. (1992) *Biochem. J.* **284,** 1–13.
32. Hansford, R. G. (1983) *Biochim. Biophys. Acta* **726,** 41–80.
33. Esterbauer, H., Schaur, R. J. & Zollner, H. (1991) *Free Radical Biol. Med.* **11,** 81–128.
34. Chen, J. J., Bertrand, H. & Yu, B. P. (1995) *Free Radical Biol. Med.* **19,** 583–590.
35. Siems, W. G., Hapner, S. J. & van Kuijk, F. J. (1996) *Free Radical Biol. Med.* **20,** 215–223.
36. Szweda, L. I., Uchida, K., Tsai, L. & Stadtman, E. R. (1993) *J. Biol. Chem.* **268,** 3342–3347.
37. Uchida, K. & Stadtman, E. R. (1993) *J. Biol. Chem.* **268,** 6388–6393.
38. Perham, R. N. (1991) *Biochemistry* **30,** 8501–8512.
39. Barlow, C. H. & Chance, B. (1976) *Science* **193,** 909–910.
40. Barlow, C. H., Harken, A. H. & Chance, B. (1977) *Ann. Surg.* **186,** 737–740.